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Genetic and phylogenetic characterization of the type II cyclobutane pyrimidine dimer photolyases encoded by Leporipoxviruses

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Abstract

Shope fibroma virus and myxoma virus encode proteins predicted to be Type II photolyases. These are enzymes that catalyze light-dependent repair of cyclobutane pyrimidine dimers (CPDs). When the Shope fibroma virus S127L gene was expressed in an *Escherichia coli* strain lacking functional CPD repair pathways, the expressed gene protected the bacteria from 70–75% of the ultraviolet (UV) light-induced cytotoxic DNA damage. This proportion suggests that Leporipoxvirus photolyases can only repair CPDs, which typically comprise ~70% of the damage caused by short wavelength UV light. To test whether these enzymes can protect virus genomes from UV, we exposed virus suspensions to UV-C light followed by graded exposure to filtered visible light. Viruses encoding a deletion of the putative photolyase gene were unable to photoreactivate UV damage while this treatment again eliminated 70–90% of the lethal photoproducts in wild-type viruses. Western blotting detected photolyase protein in extracts prepared from purified virions and it can be deduced that the poxvirion interior must be fluid enough to permit diffusion of this ~50-kDa DNA-binding protein to the sites where it catalyzes photoreactivation. Photolyase promoters are difficult to categorize using bioinformatics methods, as they do not obviously resemble any of the known poxvirus promoter motifs. By fusing the SFV promoter to DNA encoding a luciferase open reading frame, the photolyase promoter was found to exhibit very weak late promoter activity. These data show that the genomes of Leporipoxviruses, similar to that of fowlpox virus, encode catalytically active photolyases. Phylogenetic studies also confirmed the monophyletic origin of poxviruses and suggest an ancient origin for these genes and perhaps poxviruses.

Keywords: Cyclobutane pyrimidine dimer; DNA repair; CPD photolyase; Shope fibroma virus; UV light; Myxoma virus; Virus phylogeny

Introduction

Cyclobutane pyrimidine dimers (CPD) are the most abundant form of DNA damage produced by shortwave ultraviolet (UV-C) light. They typically comprise 70–80% of the lesions formed in vivo and are cytotoxic if not efficiently repaired. Although several complementary enzymatic pathways have been identified which can catalyze the repair of CPD damage, the most elegant of these is a process called enzymatic photoreactivation. In this reaction, photon energy is trapped by enzyme-bound chromophores and used to return a CPD lesion to its two component bases (reviewed in Sancar, 2000). *Escherichia coli* and *Saccharomyces cerevisiae* photolyases were the first such enzymes to be biochemically characterized and cloned, and the structure of the *E. coli* enzyme (Brash et al., 1985) has been reported. Subsequent research has shown that CPD photolyases are widely distributed throughout Archaea, Eubacteria, and Eukaryota taxa including such chordates as bony fish and marsupials. However, aside from recent reports of transgenic animals (Schul et al., 2002), it is generally recognized that placental mammals cannot photoreactivate CPD lesions and must therefore depend upon nucleotide excision repair

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pathways. Phylogenetic analysis suggests that the CPD photolyases belong to a large and ancient gene family which also includes blue-light receptors in plants and humans, and enzymes catalyzing the photoreactivation of UV-induced [6-4] pyrimidine-pyrimidone adducts (Kanai et al., 1997). Two forms of photolyases have been distinguished based upon primary sequence differences and termed types (or classes) I and II.

In contrast to cellular organisms, the genomes of viruses and phage provide little space for the inclusion of UV-repair genes, and for many years the only known gene was *den*V, which encodes the CPD-specific *N*-glycosylase of bacteriophage T4 (McMillan et al., 1981). This situation has changed in recent years with the complete sequencing of a number of large DNA viruses. The list of virus-encoded UV repair genes now includes a chlorella virus-encoded CPD *N*-glycosylase (McCullough et al., 1998) and an African swine fever virus DNA polymerase β ortholog that may play a role in base excision repair (Oliveros et al., 1997). This list might also be considered to include the DNA ligases encoded by many Chordopoxviruses. These poxviral gene products resemble DNA ligase III and, similar to cellular ligase III, have been shown to play a role in UV repair (Kerr et al., 1991; Parks et al., 1998).

In addition to these DNA repair enzymes, it has also been discovered through genome sequencing efforts that many poxviruses encode type II DNA photolyases. Viruses known to encode these genes include several Entomopoxviruses (Afonso et al., 1999; Bawden et al., 2000), as well such as Chordopoxviruses as the Avipoxvirus fowlpox (Afonso et al., 2000), and the Leporipoxviruses myxoma (MYX) and Shope (or rabbit) fibroma virus (SFV) (Cameron et al., 1999; Willer et al., 1999). That these enzymes can promote UV repair was recently demonstrated by Srinivasan et al., who showed that fowlpox virions can be photoreactivated by visible light and that open reading frame FPV158 encodes a gene capable of complementing an *E. coli* photolyase deficiency (Srinivasan et al., 2001). Here we show that the proteins encoded by SFV and MYX viruses are also functional photolyases and provide additional insights into the substrate specificity, expression kinetics, and distribution of these enzymes. The fact that viruses which now parasitize mammals are shown here to encode functional CPD photolyases raises intriguing questions concerning the evolutionary origin of these enzymes. The acquisition of photolyase genes by SFV and myxoma viruses almost certainly long predates the evolutionary origin of their rabbit hosts.

Results and discussion

Leporipoxvirus class II CPD photolyase orthologs

The sequencing of SFV and MYX virus led to the discovery of a new 1.3-kb open reading frame sandwiched between Leporipoxvirus homologs of the vaccinia A37R and A38L genes (Fig. 1a) (Cameron et al., 1999; Willer et al., 1999). The two viruses encode very similar proteins (85% amino acid identity over 445 residues) and for the purposes of this communication we have treated them as being functionally equivalent. The MYX gene (M127L) encodes a 52.4-kDa protein that was tentatively identified as an early gene product based upon the promoter sequence (Cameron et al., 1999). The SFV gene (S127L) encodes a 52.5-kDa protein associated with upstream sequence features that do not clearly match any of the usual early, intermediate, and/or late poxviral promoter motifs (Willer et al., 1999). This may not be very surprising, as photolyases are often expressed at low levels and thus photolyase promoters might not be so amenable to categorization as would more highly utilized poxvirus promoters. Two copies of an early transcription termination motif (Yuen and Moss, 1987) were also detected within the N-terminal coding portion of the SFV gene (Fig. 1), but as we have noted previously, this feature does not reliably preclude S127L being an early gene (Willer et al., 1999).

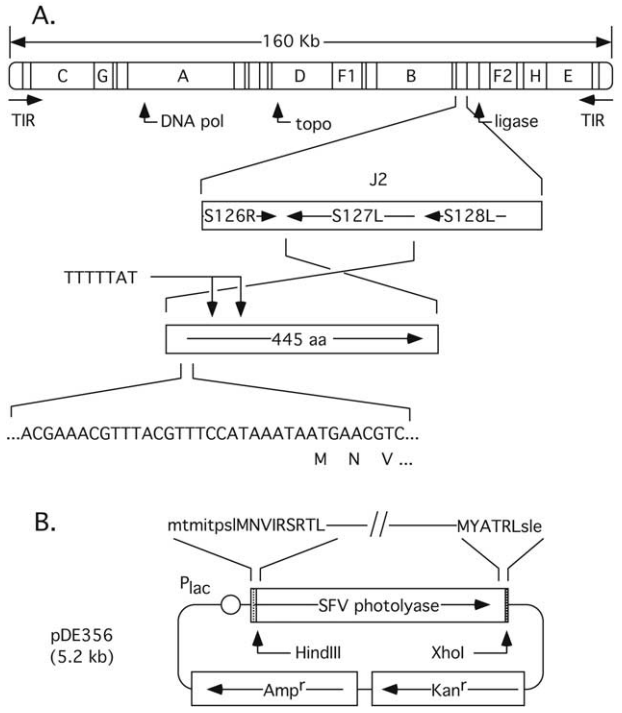

Fig. 1. Physical map and DNA sequence features of the gene encoding SFV CPD photolyase. (A) The BamHI restriction map of SFV (strain Kasza) is presented. SFV photolyase is located in the middle of the BamHI J2 fragment, flanked by SFV homologs of the vaccinia virus A37R and A38L (LAP-like protein) genes. The locations of sequences matching the early gene transcription termination motif, T_5NT, are also indicated. S126R is predicted to be an early gene, but one cannot predict the kinetics of S127L and S128L expression from sequence analysis alone (Willer et al., 1999). (B) Recombinant bacterial expression plasmid used in this study. Only the protein-coding elements are drawn to scale along with the unique restriction sites used in cloning the photolyase. SFV and vector-derived amino acids are shown in upper and lower case, respectively.

We used a variety of methods to try and gain some insights into the pattern of expression of these proteins. Northern blot and reverse transcriptase coupled PCR analysis generated data that were difficult to interpret. This was due to the presence of an abundance of read-through transcripts that could not be differentiated from bona fide photolyase transcripts. We were also unable to detect photolyase-specific transcripts using ^{32}P-labeled antisense primers, reverse transcriptase, and mRNA extracted from cells infected with wild-type or photolyase deleted viruses (see below). Finally, attempts to specifically immunoprecipitate ^{32}S-labeled photolyase protein at different times from virus-infected cell lysates were also unsuccessful.

These data suggested that Leporipoxvirus photolyase promoters are not very active and that another more sensitive method would be needed to detect gene expression. We therefore constructed a plasmid encoding a luciferase gene cassette under the regulation of the SFV photolyase promoter and transfected this DNA into SFV-infected cells. [The longest SFV promoter motifs are about 18 nt long (Willer et al., 1999), so 30 nucleotides were copied from upstream of the photolyase start codon to capture even a hybrid early-late promoter.] For comparison purposes, we also transfected SFV-infected cells with plasmids encoding the luciferase gene under the regulation of a strong-late P11K vaccinia virus promoter (pRP406). Luciferase activity was then measured at different times postinfection. The results of these experiments are shown in Fig. 2.

Late gene expression in SFV-infected cells starts about 10–12 h postinfection and this was consistent with the timing of P11K-regulated luciferase gene expression (Fig. 2). The SFV photolyase also appears to be a late gene because the timing of luciferase expression, driven from this promoter, was essentially identical to the timing of P11K-regulated activity (Fig. 2). Control experiments, using a promoterless luciferase construct, showed that this expression was dependent upon sequences encoded upstream of the S127L open reading frame (Fig. 2, negative control). This late pattern of gene expression would also be consistent with the presence of the two early gene transcription termination motifs within the gene and parallels the late gene expression of fowlpox photolyase (Srinivasan et al., 2001). What was very different, however, was the relative level of expression. The photolyase promoter produced only 1/2000th of the amount of luciferase expressed from a strong late promoter and similar data were reported for the fowlpox gene (cited in Srinivasan et al., 2001). It has been estimated that the natural abundance of *E. coli* photolyase amounts to only 10–20 molecules per cell (Harm et al., 1968). The SFV photolyase promoter may be the worst poxvirus late promoter yet described and yet little photolyase expression probably still also suffices to meet the needs of these viruses. We have not examined the kinetics of expression of MYX M127L and the promoter is not identical to that of SFV. However, the conservation of one of the two N-terminal T_5NT motifs, within the open reading frame, suggests that M127L may also be regulated by a weak late promoter.

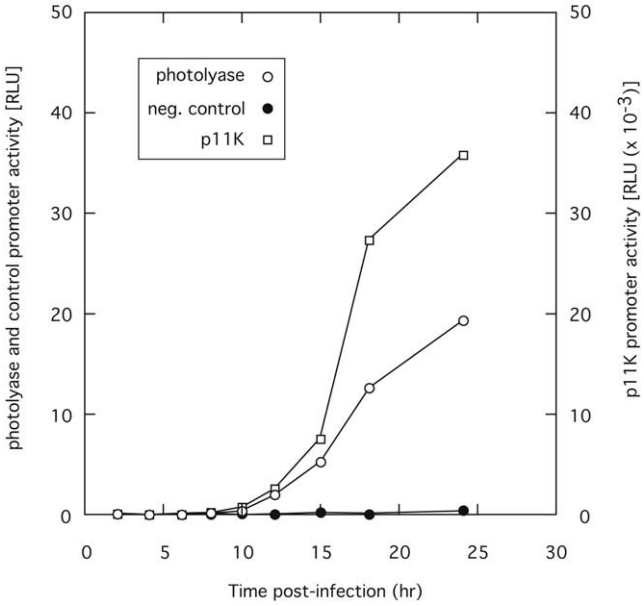

Fig. 2. Kinetics of luciferase expression regulated by the S127L promoter in SFV-infected cells. SFV-infected BGMK cells were transfected with plasmids encoding a luciferase gene under the regulation of either the S127L photolyase promoter (—○—), a vaccinia virus P11K promoter (—□—), or a promoterless construct (—●—). Cells were cultured at 37°C, harvested at the indicated times, and assayed for induced luciferase activity. Note the 1000-fold difference in scales. RLU = relative light units.

SFV photolyase complements UV-repair defects in E. coli

To test whether Leporipoxvirus photolyases are catalytically active proteins, we first tried to prepare recombinant SFV photolyase using both bacterial pET vectors and vaccinia T7-based expression vectors (Fuerst et al., 1986). Unfortunately, we could not detect *any* recombinant protein production by either immunoblotting, SDS–PAGE analysis, or screening for an activity that would eliminate T4 UV-endonuclease sensitive sites from UV-irradiated plasmids (Lapointe et al., 1996). We therefore tried an alternative genetic approach, reasoning that since only a few molecules of bacterial photolyase are required per cell to create UV resistance in *E. coli*, it still might be possible to achieve these low levels of protein expression with SFV photolyase. The open reading frame was cloned into a *lac*I-regulated *E. coli* expression vector, plasmid pDE356, which was then transformed into *E. coli* strain CSR603 (Fig. 1B). Mutations in this strain inactivate the UvrABC, RecA, and photolyase-dependent repair pathways and thus CSR603 is exquisitely sensitive to UV damage. Transformed cells were treated with isopropylthio-β-D-galactoside to induce protein synthesis, plated, and then exposed to 0 or 1.1 J/m^2 254 nm UV light. In the absence of photoreactivating light, this UV dose

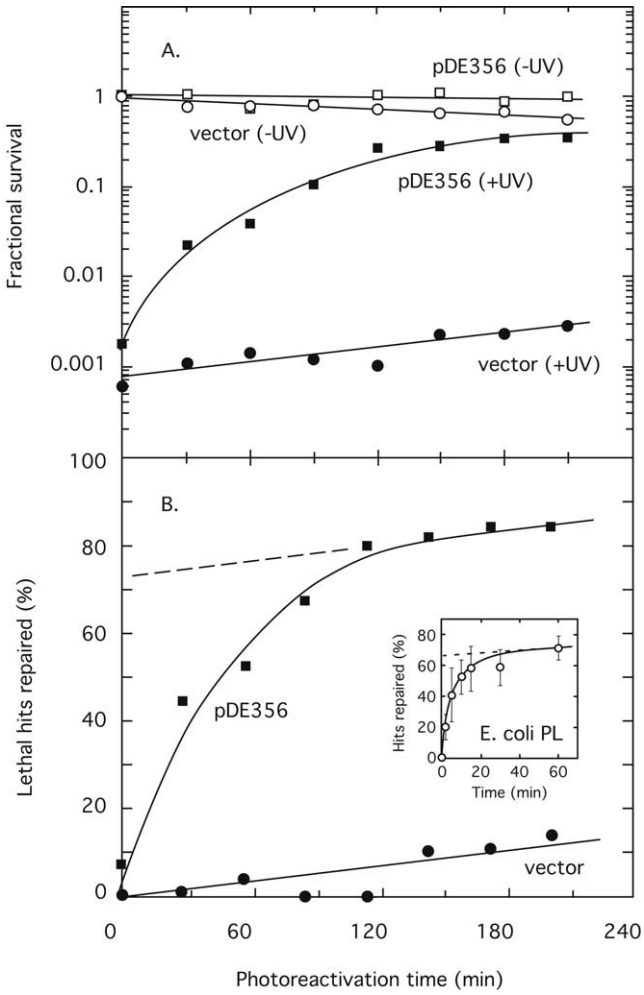

Fig. 3. Complementation of E. coli UV-repair defects by SFV photolyase. (A) Strain CSR603 was transformed with either a plasmid encoding SFV photolyase (pDE356; —■—, —□—)or a vector control (pCR2.1; —●—, —○—). After induction of protein synthesis, the cells were diluted, plated on kanamycin-containing media, and exposed to 0 (—□—, —○—) or 1.1 J/m^2 (—■—, —●—) UV light followed by the indicated dose of fluorescent light. After overnight incubation the fractional survival at each time point was calculated relative to the number of colonies formed in the absence of any light treatment. (B) The data presented in (A) was transformed mathematically, as described under Materials and methods, to determine the number of lethal hits repaired in either strain. Shown (inset) is the reactivation kinetics observed in *E. coli* strain CSR603 carrying a cloned copy to the *E. coli phr* gene.

results in 0.1% survival and thus creates an average of seven lethal "hits" per cell assuming that the damage is Poisson distributed (Fig. 3A). UV-treated (or control) cells were then held at room temperature for 3.5 h during which time they were exposed to varying amounts of UV-filtered fluorescent light. The visible light treatment had little or no effect on non-UV-treated cells regardless of the transforming plasmid. However, bacteria transformed with the plasmid encoding SFV photolyase catalyzed light-dependent removal of 70–75% of the cytotoxic UV lesions over a 2-h period (Fig. 3B). In contrast, cells transformed with the vector plasmid alone demonstrated only the residual non-enzymatic photoreactivation activity seen in *phrB*$^-$ strains (Husain and Sancar, 1987). We concluded that the 1.3-kb open reading frame encodes a functional photolyase which can catalyze light-dependent repair of UV damage. Moreover, *E. coli* can provide at least some of the chromophore(s) assumed to be essential for activity. At a minimum, this chromophore would likely be $FADH_2$, since it is essential for catalysis. Moreover active recombinant *Arabidopsis* and *Potorous* type II photolyases can be recovered from *E. coli* incorporating just an $FADH_2$ cofactor (Kleiner et al., 1999). Whether poxvirus photolyases utilize a second chromophore, such as the 5,10-methenyltetrahydrofolate found in *Drosophila* type II photolyase remains to be established.

A notable feature of this reaction is that no more than ~75% of the lethal damage induced by ultraviolet light could be enzymatically eliminated by the recombinant protein, which provides important insights into the substrate specificity of SFV and poxvirus photolyases. UV light creates a spectrum of DNA damage, with cyclobutane pyrimidine dimers and [6-4] pyrimidine-pyrimidone photoproducts being the two predominant types of UV-induced lethal lesions. These lesions are typically formed in a 2:1 ratio of CPD to [6-4] adducts when *E. coli* is exposed to 254 nm UV light (Chandrasekhar and Van Houten, 2000) and since the *E. coli* enzyme efficiently repairs only the CPD adduct (Brash et al., 1985), only ~70% of such adducts can be repaired by *E. coli* photolyase [Fig. 3B (inset)]. The fact that SFV photolyase similarly repairs only 70–75% of the lethal damage introduced by UV-C light is consistent with the prediction that similar to the *E. coli* enzyme, and similar to other Type II photolyases, it can recognize and repair only CPD lesions.

Leporipoxvirus photolyases reverse DNA damage in UV-irradiated virions

The biological advantages of a virus-encoded photolyase seem obvious. Such an enzyme is expected to provide some defense against UV radiation. To test whether Leporipoxvirus photolyases can, like their fowlpox homologs, protect virus particles from UV damage, we exposed a virus suspension to UV light and then titered plaque forming efficiency following exposure to graded doses of photoreactivating light. A strain of MYX virus was used in these studies, in which the *E. coli* β-galactosidase gene had been inserted near the M009L gene (Opgenorth et al., 1992), since this "MYXlac" virus is more easily and accurately titered than with wild-type MYX virus or SFV. Results summarizing a series of such experiments are presented in Fig. 4. Low doses of UV inactivated MYXlac viruses with simple log-linear kinetics (data not shown). In the absence of exposure to photoreactivating light, 25 J/m^2 of UV-C light reduced the virus titer by about 70%, introducing approximately 1.3 lethal hits per virus genome under conditions which would introduce four to five CPDs into puri-

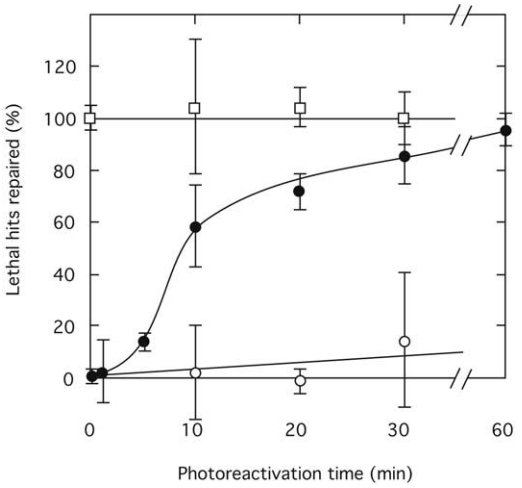

Fig. 4. Photoreactivation of UV-irradiated myxoma virus. A suspension of MYX1ac virus was treated with 0 or 25 J/m^2 of UV-C light and then exposed to timed doses of filtered photoreactivating light. The virus titer was then determined by plating on SIRC cells under conditions that minimized further light exposure. UV-treated control viruses were placed under the photoreactivating lamps while wrapped tightly in aluminum foil. The graph summarizes data obtained from three independent experiments with each titer typically measured in duplicate. Error bars show standard deviations. Legend: UV-treated and photoreactivated virus (—●—); UV-treated virus, mock photoreactivated (—○—); non-UV treated virus exposed to photoreactivating light (—□—).

fied DNA (Douki and Cadet, 2001). Subsequent exposure to varied doses of filtered blue light reversed 80–100% of these lethal hits (Fig. 4). In UV-C-irradiated mammalian cells, CPD:[6-4] adducts are reportedly generated in a 4:1 ratio (Perdiz et al., 2000) so Leporipoxvirus photolyases again appear to photoreactivate roughly that portion of the UV damage which corresponds to CPD lesions, regardless of whether that damage is located in bacterial DNA or in packaged virus genomes. Although the inability to repair [6-4] photoproducts may seem an undesirable biological property, in reality sunlight produces relatively less [6-4] lesions in mammalian cell DNA than does 254 nm UV-C (Perdiz et al., 2000) and thus the enzyme specificity is less of a problem for poxviruses under natural conditions.

Excited singlet $FADH_2$ is too unstable to permit photolyases to "store" photon energy for any significant length of time and so only when bound to a CPD can these enzymes catalyze photolysis (Kim et al., 1992). Therefore the CPD repair we detect must occur during the exposure of virus particles to light, and not following the subsequent infection and uncoating steps that were performed in the dark. An interesting conclusion that can then be drawn from these observations is that the interior of poxvirions must be sufficiently fluid to permit the diffusion of particles the size of photolyases along the virus chromosome and to the site of a CPD, during the several minutes separating UV- from visible light-exposure. One would also assume that the enzyme should be active in infected cells. We tried to detect photoreactivation activity in virus-infected cells without success, but there are practical technical problems associated with exposing mammalian cells to intense visible light, which render suspect any conclusions we can draw from these experiments.

Myxoma virions encapsidate photolyase antigen

These studies suggest that it is the photolyases packaged in mature virions which catalyze photoreactivation. It seems unlikely that this activity could be derived from the host since no placental mammal has been shown to date to encode a photolyase. Nevertheless, to investigate the distribution and origin of the photolyase activity, we used Western blotting to follow the location of the antigen in gradient-purified virus particles. A fusion protein was prepared in *E. coli* comprising part of the MYX virus M127L open reading frame linked in-frame with glutathione-*S*-transferase, and this recombinant protein was used to raise a polyclonal antibody in rabbits. This antibody was then further affinity purified by binding it to and eluting it from recombinant photolyase antigen. Using this reagent, an ~51-kDa antigen was detected in crude extracts of virus-infected cells along with a number of smaller infection-dependent polypeptides, which may be protein degradation products (Fig. 5). The mass of the largest of these polypeptides correlates well with the 52.4-kDa mass predicted for the M127L-encoded protein and this protein seemed to be selectively encapsi-

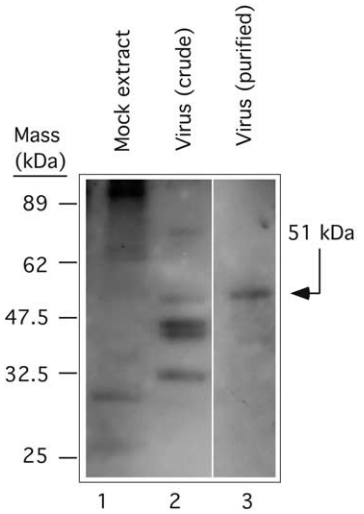

Fig. 5. Western blot analysis of myxoma virus photolyase distribution. Extracts were prepared from either mock-infected cells (lane 1), crude virus suspensions purified using differential centrifugation (lane 2), or virus particles purified by sedimentation through sucrose gradients (lane 3). Samples were boiled in loading buffer, separated using 12% polyacrylamide gels, Western blotted, and detected using an affinity-purified photolyase-specific antibody. Myxoma photolyase is predicted to have a mass of 52.4 kDa. A mix of full-length and putative degradation products are detected in infected cells; only the full-length polypeptide is virion associated. The composition of the crude and purified virus specimens differs greatly, so the small difference in the electrophoretic mobility of the full-length proteins, in lanes 2 and 3, is of questionable significance.

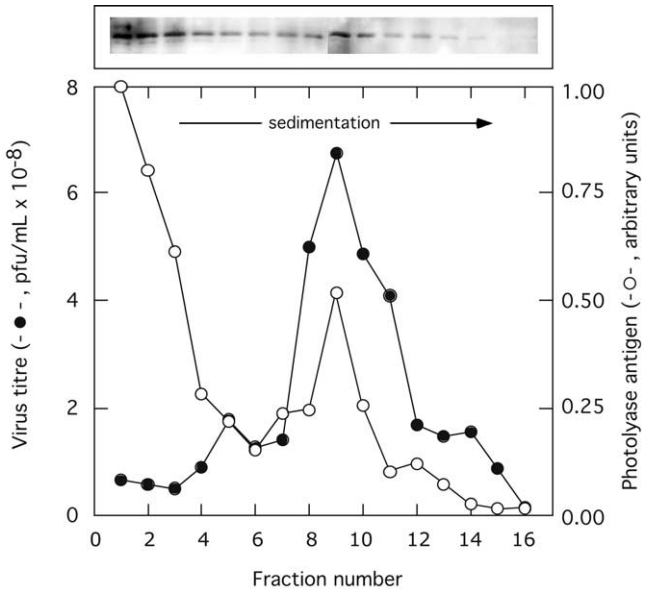

Fig. 6. Distribution of photolyase antigen-in gradient-purified myxoma virus particles. Approximately 10^{10} PFU of partially purified MYXlac virus particles were applied to the top of a 24–40% sucrose gradient and centrifuged for 50 min at 40,000 *g* in an SW41 rotor. The tube was fractionated by bottom puncture and each fraction was sampled to determine the virus titer. Each fraction was also subjected to Western blot analysis as described in Fig. 5 except that the protein was detected using unpurified photolyase-specific antibody. Densitometry was used to estimate the intensity of the chemiluminescent signal.

dated in gradient-purified particles (Figs. 5 and 6). Although this method cannot further locate M127L on the exterior or interior of a virus particle, virus DNA was shown to be photoreactivated by such an enzyme (Fig. 4) and DNA resides inside a virion. Therefore, this combination of genetic and immunological evidence shows conclusively that MYX virus incorporates MYX photolyase within the interior of virus particles.

Deleting the photolyase gene blocks virus photoreactivation

A gene knockout experiment was also performed to formally prove that the M/S127L genes encode photolyases. A DNA cassette encoding the *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene (Falkner and Moss, 1988) was assembled, flanked by 990- and 900-bp sequences homologous to sequences flanking the MYX M127L gene. This DNA was transfected into MYX-infected cells and recombinant viruses, resistant to mycophenolic acid, were isolated and plaque purified. PCR and Southern blot analyses showed that these recombinant viruses encoded a precise deletion of the M127L open reading frame and its replacement by the gpt cassette (data not shown). These knockout viruses were then exposed to UV light and the photoreactivation capacity was measured as previously described. As predicted, photolyase deficient viruses were unable to photoreactivate UV damage (Fig. 7).

Phylogeny of poxvirus photolyases

We have previously described the extraordinary conservation of amino acid sequence between Leporipoxvirus photolyases and the other Type II enzymes (Willer et al., 1999). This degree of sequence conservation is unusual, because rapid sequence drift tends to obscure the relationship between most virus genes and their cellular homologs. SFV and MYX viruses encode the smallest known photolyases, and more than a third of the 445 amino acids are absolutely conserved between proteins encoded by organisms as different as poxviruses, plants, and opossums. We used the fact that one can unambiguously align almost all of M/S127L with homologous portions of other CPD photolyases, to investigate the relationships between these enzymes and to address two questions. First, do photolyase phylogenies further support the contention that poxviruses form a monophyletic group (Iyer et al., 2001) and, second, can these analyses provide any insights into the origin(s) of poxviruses and other large DNA viruses?

Two different software tools were used to reconstruct photolyase phylogenies. The first uses Bayesian methods to infer phylogeny (“MRBAYES”); the second uses the neighbor-joining method (Saitou and Nei, 1987), which clusters sequences based upon overall similarity (“PHYLIP”). Interestingly both methods generated nearly identical topologies of photolyase phylogeny, with the only inconsistency being where to best locate the *Drosophila melanogaster* photolyase (Fig. 8). Other studies using maximum likelihood methods replicate the neighbor-joining phylogenetic tree (Kanai et al., 1997). The proposed clustering of poxvirus genes with animal type II photolyases (Srinivasan et al., 2001) was unfortunately not well supported statistically by either method. In our analysis, poxvirus photolyases formed

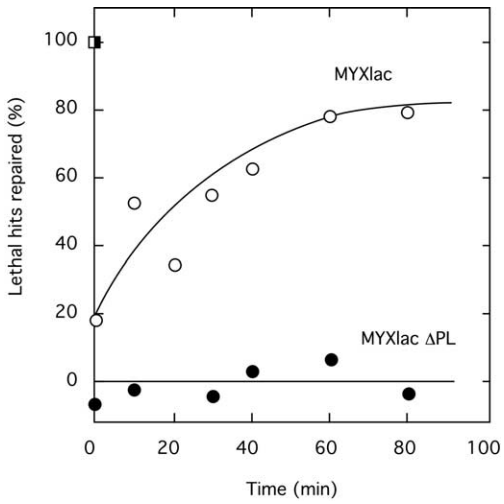

Fig. 7. Photoreactivation of photolyase knock-out virus. Wild-type (MYX1ac) or photolyase-deficient viruses (MYXlac ΔPL) were UV-irradiated and assayed for photoreactivation capacity as described in Fig. 4. UV-irradiated virus (—●—, —○—), non-UV-treated virus controls (—■—, —□—).

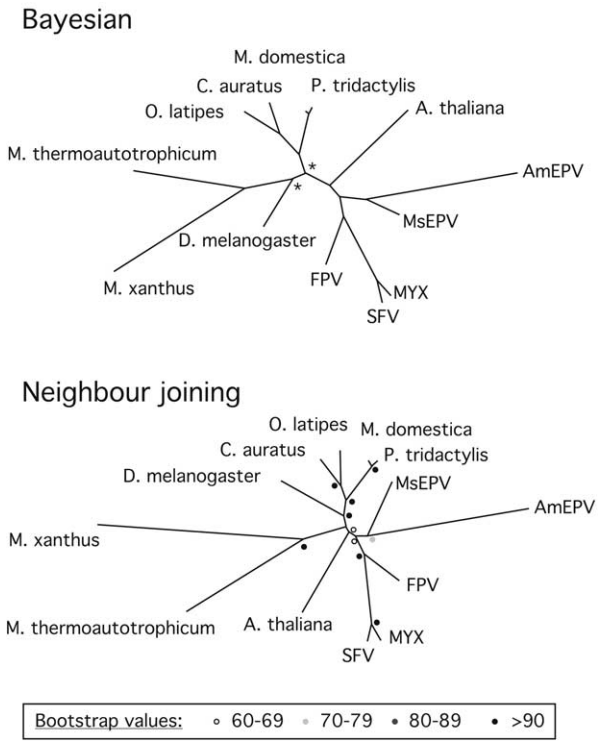

Fig. 8. Phylogeny of poxvirus and other type II photolyases. Class II CPD photolyases were originally defined as a group incorporating *D. melanogaster, Oryzias latipes, Carassius auratus, Potorous tridactylis, and Methanobacterium thermoautotrophicum* proteins (Yasui et al., 1994) and has been expanded here to incorporate a selection of more recent discoveries. (Top) Unrooted phylogenetic tree determined using a Bayesian method. All cluster frequencies were 100% except those indicated (*), which were 91% (Bottom) Unrooted phylogenetic tree determined using the neighbour-joining method. Bootstrap probabilities are indicated where greater than 60% (inset). MsEPV, *Melanoplus sanguinipes* entomopoxvirus; AmEPV, *Amsacta moorei* entomopoxvirus; FPV, fowlpox virus; MYX, myxoma virus; SFV, Shope fibroma virus.

a single clade within which the traditional distinction between Entomopoxviruses and Chordopoxviruses is readily apparent. The presence of this cluster reinforces a view gained from studying other conserved genes: that poxviruses probably do form a monophyletic group. That is, Entomopoxviruses and Chordopoxviruses probably derive from a common ancestor and not through convergent evolution. A noteworthy feature of this clade is that all of the affiliates are insect-vectored poxviruses. It is tempting to speculate that, while this transmission mode has some obvious advantages, it may also expose insect-vectored viruses to enough inactivating UV light to have long favored the retention of a photolyase gene. In contrast, those viruses that are distributed by other mechanisms may not accumulate enough UV damage to maintain such an activity. What is perhaps most interesting is the point of origin of this group, where the branch appears to reach very far back in time with *Arabidopsis thaliana* type II photolyase perhaps forming the sister group to the poxvirus cluster. What this ancestor or gene source might have been is impossible to say given the limited power to resolve deep branches and the confounding problems created by horizontal transfer and gene accretion. Viruses are not necessarily as old as their genes. However, the data show that poxvirus photolyases, and by inference possibly poxviruses, have an origin dating back as far as the divergence of plants, animals, and fungi, approximately one billion years ago (Doolittle et al., 1996).

Materials and methods

Cells, viruses, and virus culture

Rabbit corneal fibroblast (SIRC) cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 5% fetal bovine serum. Myxoma strain MYXlac was described previously and encodes the *E. coli lac*Z gene inserted at the intergenic locus between the MYX growth factor gene and M009L (Opgenorth et al., 1992). MYXlac virions were purified by sedimentation through sucrose gradients.

Cloning of recombinant Leporipoxvirus and E. coli photolyases

The SFV photolyase open reading frame was cloned from pKBJ2 (Delange et al., 1984) using the PCR and two oligonucleotide primers (5′ CGAAGCTTGATGAACGTCATACGTAGTAGACA 3′ and 5′ ACTCGAGCGACAGTCGAGTAGCATACATCTTG 3′). The resulting fragment was "Topo-cloned" into pCR2.1 (Invitrogen) and transformed into strain XL-1 Blue. A mutation-free clone was identified by DNA sequencing and then treated with *Hin*dIII and DNA ligase to eliminate 21 polylinker-encoded N-terminal amino acids. The resulting plasmid (pDE356, Fig. 1) was retransformed into strain CSR603 (AB1157 *gyrA*98 *uvrA*6 *recA*1 *phrB*1). This recombinant plasmid did not permit efficient expression of full-length SFV photolyase protein in *E. coli.* To prepare sufficient antigen for antibody production, a portion of the MYX M127L gene (encoding amino acids 137–328) was subsequently cloned using the PCR and two primers (5′ CAACGGTCATACAGGTGGA 3′ and 5′ TATACGTATCGTATAGATAGGGT 3′). The resulting 570-bp fragment was cloned into pCR2.1 and recloned into pGEX2T (Pharmacia). Recombinant glutathione-*S*-transferase fusion protein was prepared and affinity purified as described (Cao et al., 2002).

The PCR and two oligonucleotide primers (5′ AAGAGTCGCCGTAAGGGAG 3′ and 5′ AGTGTCGCGGGTAATCGTA 3′) were also used to clone a wild-type *phr* gene from *E. coli* strain K12λ for control purposes. The resulting 3.1-kb DNA (spanning *E. coli* nucleotides 737,669–740,778) was then cloned into pCR2.1, sequenced, and retransformed into strain CSR603. The cloned fragment encoded both the *E. coli* photolyase open-reading

frame and its native promoter, complementation thus rendered the CSR603 transformant *phr*$^+$ (Fig. 3, inset).

Luciferase assays

A 50-mer PCR primer was prepared that fused the 5′ end of a firefly luciferase open reading frame (underlined) to the S127L promoter and start codon (5′ TAAATCGGACGAAACGTTTACGTTTCCATAATAATGGAAGACGCCAAAAA 3′). A stop codon was also added upstream of the promoter and in-frame with the luciferase start codon (double-underline) to ensure that any transcripts later originating from cryptic plasmid-encoded promoters would not confound the analysis. A second 47-mer primer, encoding the 3′ end of the luciferase gene (underlined), was also prepared and used in PCR reactions to amplify the gene (5′ GTCCCATAACCACTTGCTGACGACCGATTACAATAGCTAAGAATTTC 3′). The PCR product was cloned into pCR2.1, and the resulting plasmid (pTOPO-SPhoto-luc) was sequenced to verify the structure. The same procedure was used to construct an additional control plasmid encoding the S127L open reading frame, but lacking a promoter (pTOPO-Luc-P_0). In this case, the PCR reaction used a 23-mer promoterless primer (5′ TAGGCGATGGAAGACGCCAAAAA 3′) instead of the 50-mer promoter primer noted above. Calcium phosphate was used to separately transfect pTOPO-SPhoto-luc, pRP406, or pTOPO-Luc-P_0 into SFV-infected BGMK cells, and the amount of luciferase activity was determined as described (Yao and Evans, 2001).

Complementation of E. coli UV-repair defects

Bacteria were shaken at 37°C in Luria broth (LB) supplemented with 50 μg/mL thymine and 50 μg/mL kanamycin. When the OD_{600} = 0.3, isopropylthio-β-D-galactoside was added to a final concentration of 500 μM, and the shaking was continued for 1 h. The cultures were then diluted in saline and 0.1 mL quantities were plated on LB agar containing 50 μg/mL thymine, 50 μg/mL kanamycin, and 1.5% Bacto agar. Half of the plates were exposed to an unfiltered mercury vapor lamp (Model R-52G, UVP Inc., San Gabriel, CA) for 10 s at a dose rate of 0.11 J/m^2/s. The plates were then placed 45 cm from a single fluorescent bulb (General Electric FT30T12-CW-RS) in a foil-lined chamber. The light was filtered through 2× 0.005-gauge cellulose acetate filters and the petri dish lids to eliminate UV. After the indicated times (Fig. 3), the plates were wrapped in foil, transferred to 37°C, and cultured overnight. A Poisson transform was used to calculate the proportion of lethal hits repaired. If d = the dose of photoreactivating light, L_d = average number of lethal hits per cell remaining at dose d, and f_d = the fractional survival measured at dose d, then $L_d = -\ln(f_d)$, and the proportion of hits repaired = $100\% \times (L_0 - L_d)/(L_0)$. When not exposed to photoreactivating light, the cells were handled under dim light. UV doses were calibrated using a UVX digital radiometer (UVP Inc.).

Deletion of MYX M127L

Two pairs of oligonucleotide primers were used to PCR amplify sequences flanking the left (5′ ACTAGTTCTAGAGCGGCCTCTACGACGCGAATACAT 3′ and 5′ **CGAAGAACGTTAATTAAAGTACGCTA**CACGACGGT 3′) and right (5′ **TAGCGTACTTTAATTAACGTTCTTCG**CATTCGTTTA 3′ and 5′ AGCTCCACCGCGGTGGCGGCCGTCCGCCATGATAGGTTTA 3′) sides of the M127L gene. A tripartite recombination-based cloning reaction was used to clone these DNAs into *Not*I-digested pBluescript II KS(+) (Stratagene), directed by sequences homologous to the *Not*I-cut vector (underlined), and common to the two PCR fragments (bold). The reaction consisted of *Not*I-cut pBluescript vector and the two PCR amplified DNAs and was catalyzed by vaccinia DNA polymerase as described (Willer et al., 2000). A suitable plasmid clone was identified and designated pPLF. A gpt cassette was separately PCR amplified using a pTM3 template and two primers (5′ GTGTAGCGTACTTTAATTAACTAAACGGGTCTTGAGGG 3′ and 5′ AATGCGAAGAACGTTAATTAATGGCATTTCTTCTGAGCA 3′), Topo cloned into pCR2.1, excised with *Pac*I, and cloned into *Pac*I-digested pPLF by standard methods (*Pac*I sites are double-underlined, above). The resulting plasmid (pPLFgpt) was transfected into MYXlac-infected cells and recombinant viruses were selected on media containing 25 μg/mL mycophenolic acid, 250 μg/mL xanthine, and 15 μg/mL hypoxanthine. The MYXlac ΔPL virus was purified using three rounds of plaque purification.

Photoreactivation of UV-irradiated viruses

Virus suspensions were exposed to 25 J/m^2 of 254 nm UV light at a dose rate of ~25 J/m^2/min in phosphate-buffered saline in uncovered dishes. The covers were replaced and the viruses were photoreactivated about 40 cm from a source composed of three 120 W tungsten lamps. This incandescent light was filtered through aqueous $CuSO_4$, cellulose acetate, and borosilicate glass to eliminate UV and infrared light (<1% transmission below 295 nm, λ_{max} = 400 nm). Dishes were removed at the indicated times (Fig. 4) and the virus was diluted and plated on SIRC cells in near darkness. Plaques were subsequently detected by staining for β-galactosidase activity.

SDS–PAGE and Western blot analysis

Polyclonal antibody was obtained from a commercial source (Alpha Diagnostics). Antibody production involved five biweekly injections of each of two rabbits with 200 μg of recombinant MYX fusion protein. The antisera were pooled and the recombinant protein was used to affinity

purify photolyase-specific antibodies (Harlow and Lane, 1999). SDS–PAGE (12%) was performed as described previously (Zhang and Evans, 1993) and the proteins were transferred to a PVDF membrane (Millipore). The membrane was blocked for 2 h at room temperature in phosphate-buffered saline containing 0.1% Tween (PBST) containing 3% gelatin (Bio-Rad) and then incubated with antibody for 2 h at 20°C. After six washes with PBST buffer, the membrane was probed with 1:20,000 diluted horseradish peroxidase conjugated anti-mouse IgG (Bio-Rad) and washed again six times with PBST. Immunoreactive materials were detected using a chemiluminescent detection kit (Pierce).

Phylogenetic analysis

A preliminary amino acid sequence alignment was constructed using CLUSTAL W (vl.6) (Thompson et al., 1994) and then corrected manually. This left an input alignment comprising 460 sites (amino acids plus gaps) spanning 13 taxa. The PHYLIP (v3.572) program SEQBOOT was used to generate 500 bootstrap resamplings of the sequence data. The program PROTDIST and a Dayhoff PAM matrix were used to generate a distance matrix and NEIGHBOR was then used to construct an unrooted tree of the sequence data from each bootstrap replicate (Felsenstein, 1989). The program CONSENSE was used to generate a consensus tree and calculate bootstrap values for each node on the tree. Alternatively, the program "MRBAYES" was used to create up to 200,000 generations of trees, sampling one in every 100 and using default substitution parameters (Huelsenbeck and Ronquist, 2001). After deleting the first 25–50 "burn in" trees, the remaining trees were used as input for CONSENSE, which calculated the percentage of times each cluster occurred in the set of sampled trees.

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